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Mechanical phenotyping of cells and extracellular matrix as grade and stage markers of lung tumor tissues

Valeria Panzetta [a], Ida Musella [a], Ida Rapa [b], Marco Volante [b], Paolo A. Netti [a,c], Sabato Fusco [a,*]

[a] Istituto Italiano di Tecnologia, IIT@CRIB, L.go Barsanti e Matteucci 53, Naples 80125, Italy
[b] Department of Oncology at San Luigi Hospital, University of Turin, Turin, Italy
[c] DCMIPE, University of Naples Federico II, 80125 Naples, Italy

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ABSTRACT

The mechanical cross-talk between cells and the extra-cellular matrix (ECM) regulates the properties, functions and healthiness of the tissues. When this is disturbed it changes the mechanical state of the tissue components, singularly or together, and cancer, along with other diseases, may start and progress. However, the bi-univocal mechanical interplay between cells and the ECM is still not properly understood. In this study we show how a microrheology technique gives us the opportunity to evaluate the mechanics of cells and the ECM at the same time. The mechanical phenotyping was performed on the surgically removed tissues of 10 patients affected by adenocarcinoma of the lung. A correlation between the mechanics and the grade and stage of the tumor was reported and compared to the mechanical characteristics of the healthy tissue. Our findings suggest a sort of asymmetric modification of the mechanical properties of the cells and the extra-cellular matrix in the tumor, being the more compliant cell even though it resides in a stiffer matrix. Overall, the simultaneous mechanical characterization of the tissues constituents (cells and ECM) provided new support for diagnosis and offered alternative points of analysis for cancer mechanobiology.

Statement of significance

When the integrity of the mechanical cross-talk between cells and the extra-cellular matrix is disturbed cancer, along with other diseases, may initiate and progress. Here, we show how a new technique gives the opportunity to evaluate the mechanics of cells and the ECM at the same time. It was applied on surgically removed tissues of 10 patients affected by adenocarcinoma of the lung and a correlation between the mechanics and the grade and stage of the tumor was reported and compared to the mechanical characteristics of the healthy tissue.

1. Introduction

The study of biomechanics and biophysics of cancer cells underlines the role of the components of the cellular cytoskeleton (CSK) in influencing some key functions such as cell mechanics, migration, differentiation, and neoplastic transformations [1–3]. In fact modifications to the CSK induced by external stimuli, such as chemical, topographic and mechanical gradients embedded in the extra-cellular matrix (ECM), act in concert with the tumorigenic molecular signaling to affect malignant transformations [4–6].

In comparison with healthy cells, biomechanical investigations reported some common features of many types of tumor cell lines, such as a less structured CSK [7–10] with lower cell mechanical [10–12] and cyto-adhesive properties [9,10,13,14]. All factors which, from a biophysical point of view, augment the metastatic potential of the cancer cells [3,7,9,14,15]. However, most of the investigations performed *in vitro* focused on the mechanical state of the cell (both single unit and collective), neglecting the influence of the surrounding ECM. While cancer cells are more compliant than their healthy equivalents, the tissues affected by malignant tumors generally appear stiffer than healthy ones [11]. Therefore, for a very detailed mechanical phenotyping of tumors, it is necessary to consider the cross-talk between cells and their surrounding ECM. Tissue biomechanics is the result of a self-sustaining

* Corresponding author.
E-mail address: sabfusco@unina.it (S. Fusco).

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combination of interactions generating between cells and their micro-environments: i) cells may modify the environment chemically by metalloproteinase secretion [16,17], or mechanically and topographically by generation and transmission of forces [18,19]; ii) the ECM responsively adapts and, thanks to its mechanical and topographic features, is able to induce structural modifications in the CSK and to trigger different cell functions and behaviors. This closes the very intricate and complex loop of interactions existing between cells and ECM [20]. Hence the different mechanical characteristics of cancer cells which could arise either from an imprecise mimicking of their *in vitro* micro-environment, or from an insufficient resolution of the mechanical characterization at a sub-cellular and a sub-matricial level when tested *in vivo* or *ex vivo*. In fact many techniques which are used for the mechanical phenotyping of cells and tissues, like rheology or tissue elastography, suffer from poor spatial resolution and low force sensitivity.

In this work, human surgical tissue samples of lung adenocarcinoma, removed from 10 patients, were analyzed using the multiple particles tracking (MPT) technique with the aim of mechanically phenotyping the tissues at cellular and ECM levels. A mechanical classification of the cells and the ECM of each sample was performed and compared with their healthy equivalents for all patients. Results and mechanical phenotypes were correlated to the stage and the grade of the tumor, previously assessed as part of the routine diagnostic procedure. In conclusion, we investigated and compared the ECM structure and morphology of both the tumor and the healthy tissues. This double-check mechanical characterization of *ex-vivo* biopsy tissues offers new diagnostic markers of the biophysical properties of the cells and the ECM (resolved at different spatial resolutions) and also gives new interpretative analytical points relating to cancer mechanobiology.

2. Methods

2.1. Samples

All experiments were performed in accordance with guidelines and regulations approved by the Research Ethics Committees of University of Naples and Turin. Experiments were performed on bioptic tissues from 10 patients with adenocarcinoma of the lung. One millimeter thick tumor tissue fragments (approximately 1 cm^2) were isolated from fresh surgical specimens by means of a sterile scalpel. Corresponding healthy tissue samples were collected from lung parenchyma at least 3 cm away from the tumor nodule. After retrieval, tissue fragments were immediately prepared for cryopreservation as follows: samples were washed three times in a PBS buffer and transferred in 1.8 ml cryovials containing 1.5 ml of cryopreservation solution (RPMI 1640 medium supplemented with 10% foetal bovine serum and 10% DMSO). The cryovials were maintained at −20 °C for 1 h and then stored at −80 °C.

Tissues were rapidly thawed in a 37 °C water bath and maintained in complete medium during particle tracking experiments.

2.2. Ballistic injection and particle tracking intracellular and extracellular mechanics

Carboxyl-modified fluorescent polystyrene particles (0.50 μm diameter, Polyscience, Inc.) were introduced into the bioptic tissues using a ballistic gun (Bio-Rad, Hercules, CA). Helium gas at 2000 psi was used to force a macro-carrier disk coated with particles to crash into a stopping screen. The force of collision was transferred to the particles, causing their dissociation from the macro-carrier and the bombardment of tissues. Once bombarded, tissues were washed extensively with phosphate-buffered saline (PBS, Lonza) and live cells were stained with DNA-specific dye, Hoechst 33342 (Life Technologies), at a 1:1000 dilution. Cell nuclei were stained in order to discriminate between cells and extracellular matrix (ECM) during optical microscopy analyses. The particles were considered inside the cells when they are in those zones in which the cells are packed and the contributions of the ECM can be neglected (see Supplementary Fig. 1, dotted blank square). Where this procedure was not feasible, we only considered those beads which are not more than 5 μm distant from the cell nuclei as belonging to the cells. Similarly, to consider particles inside the ECM, we took advantage of the collagen autofluorescence and looked at those zones in which nuclei are absent or their contribution is fringe (see Supplementary Fig. 1, dotted red square). After incubation, tissues were washed with PBS and the motion of intra-cellular and extra-cellular fluorescent beads was recorded for a total of 5 s at 100 frames per second (yielding a total of 500 frames per video) using a digital camera (Hamamatsu, ORCA-Flash 2.8) attached to a PC and Cam control video capture software mounted on an inverted fluorescence microscope (Olympus IX81) equipped with a fluorescent mercury lamp (Olympus U-LH100L-3). An oil immersion objective (100×, NA = 1.40) at 1.6× magnification was used for particle tracking. Videos were kept short (the total duration was 5seconds) to avoid photo-bleaching of particles. To perform experiments under physiological conditions, a microscope stage incubator (Okolab, Naples, Italy) was used to keep cells at 37 °C and 5/95% CO_2/air mixture. The total number of analyzed particles was at least 200 from more than 20 cells and regions for each sample.

Particle tracking microrheology, introduced by Tseng et al. [21], allows the monitoring of local viscoelastic properties of living cells and the extra-cellular micro-environment with a high spatio-temporal resolution, collecting and analyzing the Brownian motions of particles embedded in cytoplasm and ECM, respectively. The particle displacements were tracked from the very beginning of the videos taken of beads embedded into the cells and ECM. To generate the point tracking trajectories, an ad hoc Matlab (Matlab 7) code performed two distinct steps: firstly it detected the beads in each frame, and then it linked the points into trajectories (see Supplementary Fig. 5). Each position was determined by intensity measurements through its centroid, and it was compared frame by frame to identify the trajectory for each particle, based on the principle that the closest positions in successive frames belong to the same particle (proximity principle). Once the nanoparticle trajectories had been obtained, mean squared displacements (MSDs) were calculated from Eq. (1)

$$\langle \Delta r^2(\tau) \rangle = \langle [x(t-\tau) - x(t)]^2 + [y(t-\tau) + y(t)]^2 \rangle \quad (1)$$

where angular brackets mean time average, τ is the time scale and t the elapsed time. The particles embedded in regions with a thickness similar to or smaller than the particle diameter were excluded from the analysis (cell lamellar regions).

2.3. Tissue morphology

A Confocal Leica TCS SP5 II combined with a multi-photon laser source was used to investigate tissue morphology. To prepare them for observation under microscope the tissues were sliced. Because of the softness of the tissues they had to be embedded in Optimal Cutting Temperature (Killik, Bio-Optica). The tissues were first fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS for 20 min, rinsed twice with PBS and incubated overnight in 2 M sucrose (Sigma-Aldrich) in distilled water. Afterwards the samples were embedded in OCT, snap frozen in liquid nitrogen, and stored at −80 °C. Next, the samples were sectioned at a thickness of 10 μm using a Cryostat (Leica CM 1850 UV), then mounted on coverslips and stored in the fridge (−20 °C) until staining. For actin